

Buckwheat Leaf Meal Fat. I. Its Physical and Chemical Characteristics and the Constituents of the Water-Soluble and Unsaponifiable Fractions of the Saponified Fat

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DURING the preparation of rutin from fresh green buckwheat plant (5) or from a leaf meal made by dehydrating the green plant (5, 6, 20), a fatty fraction is obtained as a by-product. The composition of this fraction has not been reported, and since the material is available in quantity, it was examined primarily to determine whether it contains constituents of economic importance. Furthermore few reports have been made on the critical examination of fats from the tissue of green plants by recently improved methods. The results of this study are reported in two papers. This paper describes the physical and chemical characteristics of the buckwheat leaf meal fat and includes a report on the composition of the water-soluble and the unsaponifiable constituents of the saponified fat. The examination and identification of the water-insoluble constituents of the saponified fat will be described later.

The characteristics of the leaf meal fat were determined either by official methods (1) or by methods referred to in the references. The results are recorded in Table I.

TABLE I
Characteristics of Fat from Japanese Buckwheat Leaf Meal¹

Fat content, ² %	5.8
Moisture and volatile matter, ³ %	6.6
Specific gravity, 25°/25°C.	1.0823
Saponification equivalent (1)	298.7
Hydroxyl value (25)	7.6
Acid value (1)	21.3
Iodine number (1)	123.9
Reichert-Meissl value (1)	7.04
Polanski value (1)	0.41
Water-soluble acids (calc. as butyric, %), (1)	12.5
Insoluble acids, ⁴ % (1)	64.9
Unsaponifiable matter, % (30)	14.9
Chlorophyll, % (1)	2.8
Red pigment, % (28)	0.50
Carotene, ⁵ % (31)	0.30
Choline, % (10)	0.30
Ash, % (1)	1.98
Nitrogen, % (33)	1.22
Phosphorus, % (1)	0.45
Sulfur, % (26)	0.21
Magnesium, % (16)	0.13

¹ All values reported are on a moisture-free basis. Numbers in parentheses refer to literature citation.

² Benzene-soluble portion of the concentrated ethanol extract of the meal.

³ By vacuum drying to constant weight at room temperature.

⁴ Free of unsaponifiable matter but contained 28.5% petroleum ether insoluble material.

⁵ Sample of fat stored for 3 years.

Japanese buckwheat leaf meal fat was used in this investigation. It does not differ greatly in chemical composition from that obtained from Tartary buckwheat, the variety now preferred for rutin manufacture (7). This was demonstrated by spectrophotometric examination of the crude fats for polyunsaturated acids (2, 3). Table II shows the results of this exami-

nation. Conjugated trienoic and tetraenoic acids were present in each variety but only in trace amounts.

Experimental

The fat was obtained by pilot-plant extraction of 1,384 pounds (dry weight) of Japanese buckwheat leaf meal with cold ethanol (95% by volume). The solvent was evaporated completely, and the resulting mixture of rutin and fatty material was extracted with benzene. The benzene solution was filtered, the solvent was removed, and the resulting fatty material, 80.3 pounds (5.8%), was stored in tight, air-free containers until used. The leaf meal fat so obtained was dark green and at room temperature was almost solid; at 100°C. it was viscous but would pour. When boiled with water, it swelled to several times its normal volume and exhibited some tendency to emulsify.

TABLE II
Spectrophotometric Analysis of Polyunsaturated Fatty Acids in Buckwheat Fat

	Japanese	Tartary
	%	%
Linoleic acid.....	6.6	9.6
Linolenic acid.....	16.3	13.9
Conjugated dienoic acid.....	6.0	4.2
Total.....	28.9	27.7

Estimation of Red Pigment. A 1,000-gram sample of fat was dissolved in 100 ml. of absolute ethanol. An aliquot of 10 ml. was extracted with petroleum ether to remove chlorophyll. After addition of petroleum ether and sufficient water to cause separation into two layers the aliquot was extracted in a liquid-liquid extractor for 5-6 hours. The dilute alcohol layer was then extracted with chloroform for 5-6 hours. The chloroform extract was adjusted to a volume of 50 ml. and examined spectroscopically for red pigment (28).

Saponification of the Fat. One kilogram of the fat (934.1 grams, moisture free) was dissolved in 7,600 ml. of hot ethanol, and potassium hydroxide (400 grams in 400 ml. of water) was added. The mixture was refluxed for 3 hours, and the alcohol was then distilled, which required about 12 hours, amounting to a saponification period of 15 hours.

Detection of Ammonia in the Alcohol Distillate. During refluxing and removal of solvent in the saponification, the alkaline distillate was collected in an excess of 5% hydrochloric acid solution. The only volatile base identified was ammonia (24). About 1.0 gram of ammonium chloride (0.03% ammonia) was obtained from the kilogram of fat.

Constituents of the Unsaponifiable Fraction. After the removal of alcohol, the residue was dissolved in water (30), and the mixture was extracted with ether to remove the unsaponifiable constituents. This frac-

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tion, which weighed 138.8 grams (14.9%, based on the weight of fat saponified), was a deep-red unctuous mass with a faint odor of violets. Preliminary tests indicated the presence of a sterol, a paraffin-like compound, and carotene. The presence of carotene was confirmed by spectrophotometric examination, which gave a value of 1.5% for carotene (31) and about 2 to 3 times this value for xanthophylls. The loss of carotene during saponification of the fat was therefore 19.4%.

Isolation of Phytol. One hundred and thirty-four grams of the fraction were mixed with 177 grams of cottonseed oil and distilled at 10 μ in a molecular still. Two fractions were taken. Fraction 1, 22.9 grams, was collected from 100-110°. This was a yellowish viscous liquid. A portion was redistilled at 8-9 mm. and came over up to 183°.

Anal. Calcd. for $C_{20}H_{40}O$: C, 81.08; H, 13.51.

Found: C, 80.92; H, 13.4.²

The index of refraction was somewhat low, and the hydrogen number was high, indicating the presence of a small quantity of some other substance. This contaminant has not been identified.

Isolation of β -Sitosterol. Fraction 2, 39.5 grams, was collected from 160-170°. When cooled, it solidified to a waxy orange mass. It gave a strong Salkowski test. A portion, 5 grams, was dissolved in hot alcohol. When cooled, this set to a buttery mass, which was treated with 1 volume of alcohol, shaken, and filtered. The crystals, freed of most of the carotene, were repeatedly recrystallized alternately from ethyl acetate and light petroleum. White crystals were obtained melting at 132.5°; raised by recrystallization from absolute alcohol to 138°.

Anal. Calcd. for $C_{27}H_{46}O$: C, 83.87; H, 11.98.

Found: C, 83.53; H, 11.53³; $[\alpha]_D^{25} -35.5^\circ$; in chloroform, $c = 1.83$, $l = 1$, $\alpha = -0.65^\circ$.

β -Sitosterol Acetate. This was prepared by boiling the sterol with acetic anhydride under reflux for 1 hour. On cooling, white crystals separated, which were collected and dried. They melted at 121-2°, raised by recrystallization from 95% alcohol to 128-9° (corr.).

Anal. Calcd. for $C_{27}H_{44}O \cdot COCH_3$: C, 81.25;

H, 11.21. Found: C, 81.18; H, 11.07⁴; $[\alpha]_D^{25} -40.42^\circ$; in chloroform, $c = 2.35$, $l = 1$, $\alpha = -0.95^\circ$.

Tests for Stigmasterol. To 0.23 gram of the acetate in 10 ml. of ether were added 5 ml. of 5% bromine in glacial acetic acid. The solution was not immediately decolorized, nor was there any precipitate after 24 hours at room temperature. Crude acetate, 0.5 gram, recovered from the mother liquor of the sitosterol acetate preparation, was similarly treated without revealing the presence of stigmasterol.

Isolation of an Eicosanol. On standing for several days in the cold, the alcoholic mother liquor from which crude sitosterol had been crystallized deposited a quantity of orange-colored crystals. These were collected and recrystallized from ethyl acetate (Norit). They melted over a range and gave a positive Sal-

kowski test. After seven recrystallizations alternately from ethyl acetate and light petroleum, they melted at 84-85° and gave no Salkowski test. They tended to mat together on the (Büchner) filter, forming a waxy film that resembled paraffin.

Anal. Calcd. for $C_{20}H_{40}O$: C, 80.48; H, 14.08.

Found: C, 80.58; H, 13.95⁵; $[\alpha]_D^{25} + 3.77$; in chloroform, $c = 1.988$, $l = 2$, $\alpha = 0.15^\circ$.

Eicosanol Acetate. One-half gram of the purest eicosanol was dissolved in 10 ml. of acetic anhydride, 10 ml. of pyridine were added, and the mixture was heated for 2 hours on the steam bath. On cooling, the solution gelled. Water, 200 ml., was added which caused a white flocculent precipitate. This was collected and recrystallized from alcohol. The yield was 0.458 gram, which melted at 64-65.5° on slow heating.

Anal. Calcd. for $C_{20}H_{40}O \cdot COCH_3$: C, 77.64;

H, 12.94. Found: C, 77.73; H, 13.22.⁶

The Corresponding Eicosanone. The alcohol, 180 mg., was mixed with 5 grams of powdered cupric oxide and heated in a test tube fitted with an air condenser. The alcohol melted, and before the temperature reached a dull-red heat, the oxide was reduced and a sublimate mixed with a fluid collected in the condenser. The sublimate was soluble in chloroform. The fluid proved to be water. When cold, the cuprous oxide and the condenser were washed with chloroform. On evaporation of the solvent a white waxy solid with an odor of burnt paraffin remained. This was recrystallized from light petroleum. The yield was 50 mg. It melted at 60-60.5° and gave a dinitrophenylosazone, which melted at 76-77°.

Water-Soluble Constituents of the Saponified Fat. The fatty acid soaps were converted into water-soluble and water-insoluble fractions by acidifying to pH 1.6 with 1 to 4 sulfuric acid and filtering (1).

Isolation of Lactic Acid. A portion of the water solution was exhaustively extracted with ether. Evaporation of the ether gave a liquid, which, after distilling, was used to prepare p-phenyl phenacyl esters (8). Although melting point behavior indicated the presence of several esters, only one could be separated from the mixture sufficiently pure for identification. This melted at 138.5-139.2³, and the melting point was unchanged when the ester was mixed with authentic p-phenyl phenacyl lactate, m.p. 140.5°C.

Anal. Calcd. for $C_{17}H_{16}O_4$: C, 71.81; H, 5.67.

Found: C, 71.58; H, 5.90.⁷

The water-soluble constituents were separated into two fractions by steam distillation. The pH of a test sample of the distilling liquid after 110 hours was 3.4. During this operation the distillate was collected in a slight excess of alkali, and finally the solution containing the sodium salts of volatile acids was evaporated to a small volume. The presence of lactic acid in both the volatile and nonvolatile fractions was detected by paper partition chromatograms (23).

A quantitative estimation of the lactic acid in the volatile fraction (14) gave a value of 0.11% of the weight of the original moisture-free fat saponified; the amount of lactic acid in the nonvolatile fraction was 0.76%. The total in the original fat was 0.87%.

² Averages of two determinations.

³ Averages of three determinations.

⁴ Averages of two determinations.

⁵ Averages of two determinations.

⁶ Averages of three determinations.

⁷ Averages of two determinations.

Detection and Estimation of Formic Acid. A small portion of the water-soluble volatile acid fraction gave a precipitate with saturated mercurous chloride, suggesting the presence of formic acid. A quantitative estimation of the formic acid present (1) gave a value of 0.27% based on the weight of the original fat saponified.

Isolation of Acetic Acid. A small portion of the volatile acid fraction was treated with a magnesium sulfate and mercuric oxide mixture to destroy such acids as formic, lactic, and pyruvic (13). It was then possible to prepare and identify a small quantity of p-phenyl phenacyl acetate (8). This derivative, after repeated crystallization from ethanol (65% by volume), melted at 110-111°C. It showed no depression in melting point when mixed with an authentic sample of p-phenyl phenacyl acetate that melted at 111.5-112°C. The quantity of derivative obtained was insufficient for carbon and hydrogen analyses.

Inorganic Acid Constituents. The residual non-volatile acid solution gave a strong qualitative test for phosphate with magnesia mixture and an excellent "brown ring" test for nitrate with ferrous ammonium sulfate and sulfuric acid. A freshly prepared saponified fat sample, neutralized with nitric acid, clarified with Norit, and filtered, gave a positive test for chloride with silver nitrate reagent, a positive test for phosphate with ammonium molybdate reagent, and a positive test for sulfate with barium chloride reagent in acid solution. The phosphates, nitrates, chlorides, and sulfates in the saponification mixture could be responsible for the high "soluble acids" value obtained by titration (Table I).

Glycerol. The residual solution that remained after removal of the volatile acids was tested for glycerol. The tribenzoate (22) showed no alteration in melting point when mixed with an authentic sample of glycerol tribenzoate. A portion of the solution was submitted to the "direct procedure" used by Fürst (15), with the modification suggested for interfering substances. A yellow color, which gave a green fluorescence with ultra-violet light with the reagent, 2,7-dihydroxynaphthalene (15), was obtained as a positive test for glycerol.

Discussion of Results

The hydroxyl value of the fat (Table I) was high enough to suggest the presence of alcohols, hydroxy acids, and sterols. Identification of lactic acid in the water soluble fraction of the saponified fat confirmed this in part. The absence of water insoluble hydroxy acids was indicated by the negligible hydroxyl value (25) of the insoluble acid fraction. The presence of alcohol and sterol in the unsaponifiable fraction was therefore expected and was confirmed in the examination of this fraction.

The value for unsaponifiables, 14.9%, was unusually high, and examination of this fraction revealed constituents of interest. In addition to such expected constituents as carotene, xanthophylls, and phytol, β -sitosterol and one of the eicosanols were identified in the unsaponifiable fraction. Eicosanols have rarely been identified in plant material. Etard (12) isolated from alfalfa an eicosanol, medicagol, which melted at 80°; Haller (17) isolated from the wax of *Raphia ruffia* an eicosanol that melted at 80° but was not identical with that isolated by Etard. Ellis (9) iso-

lated from wheat seedlings an eicosanol that melted at 83°, which she considered different from the compound of Etard. An eicosanol that melted at 68° was found in wheat germ oil by Ichiba (19), and *d*-eicosanol-2 was identified in the wax of the timothy bacillus by Pangborn and Anderson (27). This compound melted at 62.5-63° and was dextrorotatory; the acetate melted at 35-37°. This eicosanol was also found in the wax of the avian tubercule bacillus by Reeves and Anderson (29). A series of *n*-eicosanols has been prepared by Halpern and Adams (18) and by Churchward and coworkers (4). Since these compounds were racemic mixtures, they were expected to differ in melting point from the optically active form. Churchward *et al.* prepared the corresponding ketones from their synthetic alcohols, but their melting points were lower than that of the ketone from the buckwheat eicosanol. None of the reported constants of the previously known eicosanols are the same as those we found for buckwheat eicosanol and its derivatives, so that definite identification cannot be made at this time. Specimens of eicosanols from wheat and alfalfa are being prepared for comparison.

The acid value of the fat indicates the presence of unusually large quantities of free acids. The Reichert-Meissl value and low Polenski value point to the presence of water-soluble volatile acids but little, if any, water-insoluble volatile acids. A Reichert-Meissl value of 7.79, calculated from the analytical data for the percentage of formic and lactic acids, is in close agreement with the value reported in Table I, indicating that acetic acid or any unidentified volatile acid could be present only in traces.

Undoubtedly the low chlorophyll value, 2.8% (Table I), was due to degradation during drying and extraction of the buckwheat. Analysis of a sample of fresh Japanese buckwheat plant showed 0.27% chlorophyll (Tartary, 0.30%) on a moisture-free basis. This would give a calculated value of 4.7% chlorophyll for the fat.

Spectrophotometric characterization of red pigment material in buckwheat was made by Wender, Gortner, and Inman, who give a brief account of the discovery and attempts to identify this photosensitizing material responsible for fagopyrism (32). "Red pigment" has been mentioned in previous publications from this laboratory as a contaminant of crude rutin prepared from buckwheat (5, 6, 11, 20, 28). As shown in Table I, material of this nature was also found in buckwheat fat. Saponification of the isolated pigment under conditions identical with those used in saponifying the buckwheat fat produced 45% destruction. No red pigment could be detected in the water-soluble or the unsaponifiable fractions of the saponified fat. However both the water-insoluble fraction and the petroleum ether-insoluble portion of the water-insoluble fraction of the saponified fat showed the characteristic absorption maximum of the red pigment.

The approximate quantity of lecithin in the fat, 2%, was calculated from the choline content on the assumption that buckwheat lecithin has a molecular weight of 800 and that all the choline is present in the combined form. Apparently only about a third of the lecithin in the meal (choline content, 0.048%) was extracted with cold ethanol (95% by volume). In present commercial extraction with hot dilute isopropanol (6, 20) however extraction of lecithin

may be more complete. Determinations of fat in various buckwheat meals and mares (21) support this statement.

Summary

The leaf meal fat of the Japanese buckwheat plant has been prepared, and its physical and chemical characteristics have been determined. Spectrophotometric analysis of the fat of this variety indicates that in composition it is similar to the fat of the Tartary variety, the one now preferably used for manufacture of rutin. The large quantity of unsaponifiable matter is unusual. This fraction contains carotene, xanthophylls, phytol, β -sitosterol, and an eicosanol.

Also of interest is the presence in the fat of significant quantities of lecithin when isopropanol is used for extraction. The water-soluble acidic constituents of the saponified fat consist of the organic acids formic, acetic, and lactic, and the inorganic acids phosphoric, nitric, sulfuric, and hydrochloric. Ammonia was present in the alcohol distillate collected after saponification, and glycerol was identified in the water-soluble acid fraction. Quantitative values are given for formic and lactic acids and for ammonia.

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